REMARKS/ARGUMENTS

Reconsideration and continued examination of the above-identified application are respectfully requested.

Claim 25 is pending. Claims 27 and 28 are canceled as non-elected claims. Claim 26 was previously canceled. Claim 25 has been amended to recite "a method for selecting treatments for a cancer patient comprising collecting genomic DNA from the patient, amplifying the HLA DQB1* (Major histocompatibility complex, class II, DQ beta 1) gene from the genomic DNA using an automated PCR thermal sequencer, identifying the amino acids at positions 57 and 67 of the amino acid sequence encoded by the HLA DQB1* (Major histocompatibility complex, class II, DQ beta 1) gene of the patient, determining anti-cancer immunotherapy after cancer resection to have a statistically significant probability of prolonging the cancer patient's survival, by determining that Asp is at position 57 and Val is at position 67 of the amino acid sequence encoded by the HLA DQB1* (Major histocompatibility complex, class II, DQ beta 1) gene; and selecting anti-cancer immunotherapy after cancer resection, as a treatment for the patient; wherein the cancer of the patient is stomach cancer." Support for these amendments generally, can be found in the claims as originally filed and throughout the application, for example, at pages 24, 26, 27, 29, 79, Figure 6, and Figure 17 of the present application. Support for the limitation of "amplifying the HLA DQB1* (Major histocompatibility complex, class II, DQ beta 1) gene from the genomic DNA using an automated PCR thermal sequencer," for instance, can be found at least on page 54 of Tissue Antigens, 38: 53-59, 1991, which is incorporated by reference at page 24 of the present application. By way of this amendment, the present application has also been amended to include the information fully incorporated by reference at the time of filing. Accordingly, no questions of new matter should arise and entry of this amendment is respectfully requested.

Telephone Interview

The applicant and the applicant's representatives appreciate the telephone interviews with Examiner Sims on February 3, 13, and 24, 2009. During the interview, proposed amendments to claim 25 were discussed. During the telephone interview of February 24, 2009, the applicant's representative proposed amending the claim to further recite "amplifying the HLA DQB1* (Major histocompatibility complex, class II, DQ beta 1) gene from the genomic DNA using an automated PCR thermal sequencer." The Examiner indicated that such an amendment would be helpful in addressing the rejection under 35 U.S.C. §101.

Rejection of Claim 25 Under 35 U.S.C. §112, First Paragraph

At pages 2-3 of the Office Action, the Examiner rejects claim 25 under 35 U.S.C. §112, first paragraph. The Examiner states that the step of "determining immunotherapy" is found to be new matter that is not supported by the specification. The Examiner also states that the phrase "wherein the cancer of the patient comprises stomach cancer" is new matter not supported by the specification. The Examiner states, however, that the specification does support determining anticancer immunotherapy after cancer resection for patients with stomach cancer, but not any other type of cancer. This rejection is respectfully traversed.

Without agreeing to the correctness of the Examiner's conclusions, as suggested by the Examiner, claim 25 has been amended to recite the step of "determining anti-cancer immunotherapy after cancer resection" instead of "determining immunotherapy," and to recite that the cancer of the patient is stomach cancer.

Accordingly, this rejection should be withdrawn.

Rejection of Claim 25 Under 35 U.S.C. §112, second paragraph

At pages 4-5 of the Office Action, the Examiner rejects claim 25 under 35 U.S.C. §112, second paragraph. The Examiner states that the phrase "determining what amino acids are encoded by positions 57 and 67 of the HLA DQB1 gene" is unclear. The Examiner states that it is unclear if the word "positions" refers to the position along the nucleotide sequence comprising the gene or the amino acid sequence comprising the protein. This rejection is respectfully traversed.

Claim 25 has been amended to further clarify that the amino acids are at positions 57 and 67 of the amino acid sequence encoded by the HLA DQB1* gene of the patient, as described at page 79 and Figures 90-94 of the present application.

Accordingly, this rejection should be withdrawn.

Rejection of Claim 25 under 35 U.S.C. §101

At pages 5-6 of the Office Action, the Examiner rejects claim 25 under 35 U.S.C. §101. The Examiner states that, in order to be patentable, a claimed method must include a physical transformation or recite a tie to another statutory category of invention along with a final resulting step producing a useful, concrete, and tangible result. The Examiner states that the steps recited in claim 25 read solely on mental steps not necessitating the production of a tangible result. The Examiner also states that the claim does not recite a tie to another statutory category of invention. This rejection is respectfully traversed.

Claim 25 has been amended to further define the claimed invention. Claim 25 previously and now results in a useful, concrete, and tangible result, which is selecting treatments for a cancer patient having stomach cancer. The claimed method also recites a series of physical steps,

and not just mental steps, including for example, "collecting genomic DNA…", "amplifying the HLA DQB1* (Major histocompatibility complex, class II, DQ beta 1) gene from the genomic DNA…", "identifying the amino acids…", and "…determining that Asp is at position 57 and Val is at position 67 of the amino acid sequence…." Claim 25 also recites the use of an automated PCR thermal sequencer for amplifying the HLA DQB1* (Major histocompatibility complex, class II, DQ beta 1) gene from the genomic DNA of the patient. Thus, a critical step of the claimed method is tied to "a particular machine or apparatus," in accordance with the requirements of *In re Bilski*, 545 F.3d 943, 88 U.S.P.Q.2d 1385 (2008).

Accordingly, this rejection should be withdrawn.

CONCLUSION

In view of the foregoing remarks, the applicant respectfully requests the reconsideration of this application and the timely allowance of the pending claims.

If there are any fees due in connection with the filing of this Amendment, please charge the fees to Deposit Account No. 50-0925. If a fee is required for an extension of time under 37 C.F.R. §1.136 not accounted for above, such extension is requested and should also be charged to our Deposit Account.

Respectfully submitted,

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Attachment: Article entitled, "HLA-DQB1 genotyping by a modified PCR-RFLP method combined with group-specific primers," by Nomura et al., TISSUE ANTIGENS, 1991, No. 38, pp. 53-59 (7 pgs.)

HLA-DQB1 genotyping by a modified PCR-RFLP method combined with group-specific primers

N. Nomura, M. Ota, K. Tsuji, H. Inoko. HLA-DQB1 genotyping by a modified PCR-RFLP method combined with group-specific primers. Tissue Antigens 1991: 38: 53–59.

Abstract: We previously reported a simple technique for HLA-DQB genotyping by digestion of polymerase chain reaction-amplified genes with restriction endonucleases (PCR-RFLP method). However, this method has some problems in that some heterozygotes cannot be discriminated from each other. Furthermore, concomitantly amplified product derived from the DQB2 gene by the primers used previously also obstructs precise DQB1 genotyping. To resolve these problems, we have developed two different pairs of specific primers for selective amplification of the DQB1 gene and also used restriction endonucleases which have either a single cleavage site or, alternatively, no cleavage site in the amplified DNA region, depending on the HLA-DQB1 alleles, making reading of RFLP band patterns much easier. The second exon of the DQB1 gene was selectively amplifed by DQw1 group-specific primers and/or DQw2,3,4 group-specific primers using genomic DNAs from 70 HLA-homozygous B-cell lines and 50 healthy Japanese. Of the seven DQw1-associated DQB1 alleles, six alleles could be defined by digestion of 6 restriction enzymes, although DQB1*0602 and DQB1*0603 could not be discriminated from each other because of unavailability of suitable enzymes. Similarly, all of the six DQw2,3,4-associated DQB1 alleles could be defined by digestion of 5 restriction enzymes. Using this modified PCR-RFLP method, complete DQB1 genotyping of all heterozygotes is possible except for discrimination between DQB1*0602 and 0603. Thus this method is simpler and more practical for a routine DNA typing than the PCR-SSO method or our previous PCR-RFLP method.

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Key words: histocompatibility testing – HLA-DQB1 – PCR-RFLP – genotype

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Introduction

HLA class II molecules are heterodimeric glycoproteins that play an important role in the control of immunoreactivity and in transplantation immunity (1, 2), as well as in susceptibility to autoimmune diseases (3).

Recently, genetic polymorphism in the HLA class II region has been detected by the polymerase chain reaction (PCR) followed by hybridization with sequence-specific oligonucleotide (SSO) probes (4–7). Although this PCR-SSO method permits the precise and direct analysis of allelic variations with as little as 1 ng of genomic DNA, such as a single hair for forensic medicine (8), this procedure has some disadvantages in that it necessitates many kinds of SSO probes corresponding to all alleles, as well as strict determination of washing conditions after hybridization for each probe. We previously reported a simple and rapid method for HLA-DQA1, -DPB1, -DQB and -DRB genotyping by digestion of PCR-amplified DNA with allele-specific restriction endonucleases (PCR-RFLP method) (9–11). This PCR-RFLP method is more practical and conventional than the PCR-SSO method because digested products can be visualized directly on acrylamide gels, eliminating the need for separate hybridization with multiple probes. However, the original PCR-RFLP method has some problems in that digested fragments located close to each other sometimes obstruct precise analysis of RFLP bands, and some heterozygotes cannot be discriminated from each other (12, 13). Precise discrimination of heterozygotes in the DQB1 gene is hampered by concomitant amplification of the DQB2 (DXβ) gene (14) with the primers previously used (GH28 and GH29), leading to RFLP patterns which were difficult to

interpret (11, 13). To resolve these problems, DQB1-specific amplification, particularly group-specific amplification (12, 13), as well as digestion with restriction endonucleases which do not necessitate precise analysis of fragment sizes, will be useful for simple genotyping of DQB1.

In this study, we have employed two pairs of primers, DQw1 group-specific and DQw2,3,4 group-specific, to selectively amplify the DQB1 gene without amplified product from DQB2. We have also searched for informative restriction endonucleases which have a single recognition site in some alleles but none in other alleles in the amplified segments of DQB1 genes (5, 6, 15, 16), making reading of the generated RFLP patterns much simpler. Using this modified PCR-RFLP method, complete DQB1 genotyping of all heterozygotes is possible except for discrimination between DQB1*0602 and 0603. Similar modified PCR-RFLP typing can be successfully applied to DQA1, DPB1 (M. Ota et al., submitted) and DRB1 genotyping (M. Ota et al., submitted).

Material and Methods

DNA samples

Seventy DNA samples used in this study were distributed for Southern blot analysis in the 10th International HLA Workshop. They were isolated from EBV-transformed HLA-homozygous B-cell lines which were also used for the previous study (9–11). Genomic DNAs from 50 Japanese volunteers were isolated by phenol extraction of sodium dodecyl sulfate (SDS)-lysed and proteinase K-treated cells as described previously (17).

PCR amplification

Genomic DNA (200 ng) was amplified by the PCR procedure with 2.5 units of the Taq DNA polymerase (Perkin Elmer Cetus Inc.) (18). The reaction mixture (100 µl), containing dNTPs (200 µM) and MgCl (2.5 mM), was subjected to 35 cycles of 1 min at 96°C, 1 min at 55°C and 2 min at 72°C by automated PCR thermal sequencer (Iwaki Glass

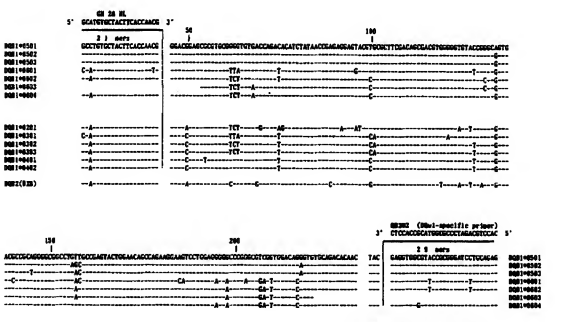

Figure 1. Alignment of nucleotide sequences of the PCR-amplified exon 2 region in the DQB1 and DQB2 (DXβ) genes from various alleles. GH28NL and QB202 primers were used for the specific amplification of the DQB1 gene with DQw1 alleles (DQB1*0501, 0502, 0503, 0601, 0602, 0603 and 0604), and GH28NL and QB204 primers are used for specific amplification of the DQB1 gene with DQw2,3,4 alleles (DQB1*0201, 0301, 0302, 0303, 0401 and 0402). The DQB2 gene cannot be amplified by using a pair of GH28NL and QB202 primers or GH28NL and QB204 primers.

Table 1.
Correlation between cleavage patterns obtained by 5 restriction endonucleases and DQw1 alleles

DQB1 allele	Group	Restriction endonucleases				
		Fok I	Apa I	Hae II	SfaN I	BssH II
0501	A	0	1	0	0	0
0503		0	1	0	0	0
0502	B	0	1	1	0	0
0601	C	1	0	0	0	0
0602	D	0	0	0	1	1
0603		0	0	0	1	1
0604	E	0	0	0	0	1

0: not cleaved; 1: cleaved.
DQB1*0501 and 0503 (Group A) can be discriminated by examining the presence of polymorphic fragments after digestion with Hph I (see Table 2).

Inc.). The second exon of the DQB1 genes with the DQw1 specificity (DQB1*0501, *0502, *0503, *0601, *0602, *0603 and *0604) was amplified by using PCR primers, GH28NL and QB202 (DQw1 group-specific primers), at 1 μM and the second exon of the DQB1 genes with the DQw2,3 or 4 specificity (DQB1*0201, *0301, *0302, *0303, *401, *0402) was amplified by using PCR primers, GH28NL and QB204 (DQw2,3,4 group-specific primers), at 1 μM (Fig. 1).

Digestion with restriction endonucleases

After amplification, aliquots (7 μl) of the reaction mixture were digested by restriction endonucleases (from 1 to 5 units) for 3 h at the optimum temperature after adding appropriate incubation buffer and distilled water up to final volume of 12 μl. Fok I, Apa I, Hae II, SfaN I and BssHII were used for digestion of the amplified DQB1 gene with DQw1 and Fok I; Bgl I and Sac I were also used for digestion of the DQB1 gene with DQw2,3 or 4. One additional restriction endonuclease (Hph I) was used for DQw1 typing and 2 more (Acy I and Hpa II) were used for DQw2, 3 or 4 typing, where necessary, for discrimination of certain alleles (see below).

Table 2.
Patterns of polymorphic fragments detected with Hph I endonuclease for discrimination between DQB1*0501 and DQB1*0503

Combinations of DQB1 alleles		Hph I (bp)	
		236	119
0501	0501	1	0
0501	0503	1	1
0503	0503	0	1

1: positive, 0: negative.

Table 3.
Correlation between cleavage patterns obtained by 3 restriction endonucleases and DQw2,3,4 alleles

DQB1 allele	Group	Restriction endonucleases		
		Fok I	Bgl I	Sac I
0201	A	1	0	0
0301				
0302	B	0	1	0
0303				
0401	C	1	1	1
0402	D	1	1	0

0: not cleaved, 1. cleaved.
DQB1*0301, *0302 and *0303 (Group B) can be discriminated by examining the presence of polymorphic fragments after digestion with Acy I and Hpa II (see Table 4).

Acrylamide gel electrophoresis

Samples of the amplified DNAs cleaved by restriction enzymes were subjected to electrophoresis using 12% polyacrylamide gel in a minigel apparatus (Mupid, Cosmo Bio Co. Ltd.). In the case of Acy I digestion, cleaved samples were subjected to electrophoresis using a 12% polyacrylamide gel in a glass plate apparatus (12 cm × 12 cm) for precise analysis of RFLP bands. Cleavage or no cleavage of amplified fragments was detected by staining with ethidium bromide.

Results

Analysis of nucleotide sequences of PCR-amplified regions in the DQw1 and DQw2,3,4 alleles of the DQB1 genes for allele-specific restriction sites

Fig. 1 summarizes the allelic variations in the 241-bp PCR-amplified region of the DQw1 alleles

Table 4.
Patterns of polymorphic fragments detected with Acy I and Hpa II restriction endonucleases for discrimination among DQB1*0301, DQB1*0302 and DQB1*0303

Combination of DQB1 alleles	Fragments digested with restriction endonucleases (bp)					
	Acy I			Hpa II		
	123	114	104	176	104	72
0301/0301	0	1	1	0	1	1
0302/0203	1	1	0	1	0	0
0303/0303	0	1	1	1	0	0
0301/0302	1	1	1	1	1	1
0301/0303	0	1	1	1	1	1
0302/0303	1	1	1	1	0	0

1: positive, 0: negative.

(DQB1*0501–0604) as well as those in the 237-bp PCR-amplified region of the DQw2,3 and 4 alleles (DQB1*0201–0402) of the DQB1 genes. The DQB2 gene (previously DXβ) cannot be amplified by using the DQw1 group-specific primers (GH28NL and QB202) or DQw2,3,4 group-specific primers (GH28NL and QB204) (data not shown).

These nucleotide sequences of the DQB1 genes were used to search for restriction endonucleases which have a single cleavage site in some alleles (indicated by 1 in Table 1) but no sites in other alleles (indicated by 0 in Table 1) in the amplified region, as established by computer analysis. Five different restriction endonucleases, Fok I, Apa I, Hae II, SfaN I and BssH II, were selected for digestion to detect DQw1 allele-specific cleavage after PCR amplification (Table 1). These 5 restriction endonucleases are expected to allow discrimination of five out of the seven DQw1 alleles, because DQB1*0501 and DQB1*0503 alleles (Table 1, Group A) as well as DQB1*0602 and DQB1*0603 alleles (Table 1, Group D) give the same cleavage patterns, although DQB1*0501 and DQB1*0503 can be discriminated by the RFLP band patterns using Hph I digestion. Digestion with Hph I gave a 236-bp fragment for the DQB1*0501 allele and a 119-bp fragment for the DQB1*0503 allele (Table 2). Unfortunately, DQB1*0602 and DQB1*0603 cannot be discriminated from each other at the moment, because no suitable restriction endonucleases recognizing a single nucleotide difference between them are available.

In a similar way, nucleotide sequences of the DQw2,3,4 alleles were used to search for restriction endonucleases specific for each of the six DQw2,3,4 alleles by computer analysis, and 3 restriction endonucleases, Fok I, Bgl I and Sac I, were selected to detect allele-specific cleavage after PCR-amplification (Table 3). Here, all of the DQw2,3,4 alleles have a cleavage site for Sac I at the 14th nucleotide position from the 3′ terminal and so, in the case of Sac I digestion, 223 bp fragment-positive

Table 5.
The cleavage patterns obtained by restriction endonucleases in the amplified DQB1 genes from healthy Japanese and the correlation between DQB1 alleles and serological typing

No. of healthy Japanese	HLA class II specificity defined by Serological typing		Cleavage patterns																Combination of DQB1 alleles
			DQw1-specific amplification							DQw2,3,4-specific amplification									
			Fok I	Apa I	Hae II	SfaN I	BssH II	Hph I	(bp)	Fok I	Bgl I	Sac I	Acy I	(bp)	Hpa II	(bp)			
	DR	DQ						236	119				123	104	176	104	72		
001	4 10	1 4	0	1	0	0	0	+	−	1	1	0							0501/0402
002	1 blank	1 blank	0	1	0	0	0	+	−										0501/0501
003	2 8	1 blank	2	0	0	2	2												0601/0602 = 0603
004	2 9	1 3	0	0	0	1	1			0	1	0	−	+	+	−	−		0602 = 0603/0303
005	2 9	1 3	1	0	0	0	0			0	1	0	−	+	+	−	−		0601/0303
006	8 blank	1 4	1	0	0	0	0			1	1	0							0601/0402
007	4 6	1 4	0	1	0	0	0	+	−	1	1	1							0501/0401
008	2 6	1 blank	0	1	0	0	0	−	+	0	1	0	−	+	−	+	+		0503/0301
009	4 6	1 3	0	1	1	0	0			0	1	0	−	+	+	−	−		0502/0303
010	1 4	1 4	0	1	0	0	0	+	−	1	1	1							0501/0401
011	4 9	3 blank								0	1	0	+	+	+	−	−		0302/0303
012	6 9	1 3	1	0	0	0	0			0	1	0	−	+	+	−	−		0601/0303
013	2 10	1 blank	2	2	0	0	0	+	+										0501/0601
014	4 blank	3 4								2	1	2	+	−	+	−	−		0302/0401
015	1 2	1 3	0	1	0	0	0	+	−	0	1	0	−	+	−	+	+		0501/0301
016	6 8	1 blank	2	0	0	0	2												0601/0604
017	2 4	1 4	1	0	0	0	0			1	1	1							0601/0401
018	1 6	1 blank	0	1	2	0	0	+	+										0501/0502
019	5 6	1 3	0	0	0	0	1			0	1	0	−	+	−	+	+		0604/0301
020	2 4	1 3	1	0	0	0	0			0	1	0	+	−	+	−	−		0601/0302

0: not cleaved, 1: cleaved, 2: both for heterozygote.
+: fragment-positive, −: fragment-negative.
One additional restriction endonuclease (Hph I) was used in DQw1-specific amplified DNA for the discrimination between DQB1*0501 and *0503. In the same manner, 2 additional restriction endonucleases (Acy I and Hpa II) were used in DQw2,3,4-specific amplified DNA for the discrimination among DQB1*0301, *0302 and *0303.

alleles were indicated by 0 for simplification in Table 3. These 3 restriction endonucleases predict discrimination of four out of the six DQw2,3,4 alleles, because DQB1*0301, DQB1*0302 and DQB1*0303 alleles (Table 3, Group B) gave the same cleavage pattern. These three alleles can be discriminated from each other by checking the size of polymorphic fragments digested with 2 additional restriction enzymes, Acy I and Hpa II (Table 4). An Acy I 123-bp fragment is positive for the DQB1*0302 allele but negative for the DQB1*0301 as well as DQB1*0303 alleles. On the other hand, a Hpa II 176-bp fragment is positive for DQB1*0302 as well as DQB1*0303 but negative for the DQB1*0301 allele, as shown in Table 4.

Digestion patterns with allele-specific restriction endonucleases in the amplified DQw1 and DQw2,3,4 alleles of the DQB1 genes from homozygous and heterozygous individuals

In order to test the method developed above, genomic DNAs from 70 HLA-homozygous B-cell lines with different DQ specificities and 50 healthy Japanese individuals were subjected to amplification of the DQB1 genes of the DQw1 and DQw2,3,4 alleles, followed by digestion with the restriction endonucleases listed above and electrophoresis in 12% acrylamide gels.

DQw1 alleles

Five out of the seven DQw1 alleles were found to be discriminated from each other on the basis of the cleavage patterns with 5 different restriction endonucleases using 70 HLA homozygous B-cell lines, as expected from Table 1. Two indistinguishable alleles, DQB1*0501 and DQB1*0503, can be discriminated by 1 additional restriction endonucleases, Hph I, as described above (Table 2). Five standard cleavage patterns of the DQw1 alleles give 10 standard heterozygous combinations, namely A/B, A/C, A/D, A/E, B/C, B/D, B/E, C/D, C/E and D/E. Note that heterozygotes should carry both cleaved (indicated by 1 in Table 1) and uncleaved (indicated by 0 in Table 1) amplified bands for cleavage pattern, as indicated by 2 in Table 5. Six out of these 10 combinations include the DQB1*0501 or DQB1*0503 allele (e.g. Group A/B, A/C, A/D and A/E in Table 1) as well as the DQB1*0602 or DQB1*0603 allele (e.g. Group A/D, B/D and C/D in Table 1) in either of the two alleles. DQB1*0501 and DQB1*0503 in the four heterozygous combinations can be discriminated from each other by checking RFLP band patterns using Hph I digestion. DQB1*0501 can be discriminated from DQB1*0503 by the presence of a 236-bp fragment no matter what allele is combined with it. In this way, 15 out of 28 homozygous and heterozygous combinations of the seven DQw1 alleles can be clearly defined, although DQB1*0602 and DQB1*0603 cannot be discriminated from each other in the remaining 13 combinations because of unavailability of any enzyme to recognize allelic variations between them, as described above.

In order to assess the possibility of DQw1-DQB1 genotyping on the basis of the cleavage patterns predicted by five standard patterns of homozygotes (Table 1) as well as 10 standard patterns of heterozygotes, genomic DNAs from 50 healthy Japanese individuals were analyzed (two examples in Fig. 2). Of the 50 DNA samples, 41 DNAs were selectively amplified by the DQw1 group-specific primer pair (GH28NL and QB202). In these 41 amplified DNAs, all of their RFLP patterns, obtained by 5

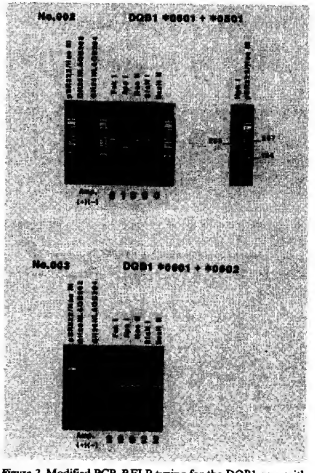

Figure 2. Modified PCR-RFLP typing for the DQB1 gene with the DQw1 alleles amplified by DQw1-specific primers (GH28NL and QB202) using DNAs of normal Japanese. Determination of their genotypes is based on the combinations given in Tables 1 and 2. Discrimination between DQB1*0602 and DQB1*0603 is impossible in DNA sample. No. 003 because of the unavailability of suitable restriction enzymes (see text). By PCR-SSO typing the genotype in this case was DQB1*0601+ DQB1*0602 (data not shown).

informative restriction endonucleases as well as 1 additional restriction enzyme, were matched with one of the combinations given in Tables 1 and 2, and their DQw1 alleles could be accurately determined and were found to be in complete agreement with those predicted from serological data (Table 5). However, five (12.2%) samples included DQB1*0602 or DQB1*0603 in either of the two-allele combinations, which, as described above, cannot be discriminated from each other by this method (data not shown).

DQw2,3,4 alleles

Four out of six DQw2,3,4 alleles were found to be discriminated from each other on the basis of the cleavage pattern with 3 restriction enzymes using 70 HLA homozygous B-cell lines, as expected from Table 3. Three indistinguishable alleles, DQB1*0301, DQB1*0302 and DQB1*0303, could be discriminated by 2 additional restriction endonucleases, Acy I and Hpa II (Table 4). Four standard cleavage patterns of DQw2,3,4 alleles give six standard heterozygous combinations, namely A/B, A/C, A/D, B/C, B/D and C/D. Three out of these six combinations include DQB1*0301, DQB1*0302 or DQB1*0303 (e.g. Group A/B, B/C and B/D in Table 3), and these three alleles can be easily discriminated from each other using additional digestion by Acy I and Hpa II, no matter what allele is combined with one of them. In this way, all 21 homozygous and heterozygous combinations of the six DQw2,3,4 DQB1 alleles can be unequivocally defined.

Genomic DNAs from 50 healthy Japanese individuals were again analyzed to assess DQw2,3,4 group-specific genotyping (two examples in Fig. 3). Of the 50 DNA samples, 39 DNAs were selectively amplified by the DQw2,3,4-specific primer pair (GH28NL and QB204). In these 39 amplified DNAs, RFLP patterns obtained by 3 informative restriction endonucleases were matched with one of the combinations given in Tables 3 and 4, and all of their DQw2,3,4 alleles could be clearly defined and again were in complete agreement with those predicted from serological data (Table 5).

Discussion

Allelic variations within the HLA-DQ subregion carry distinct allospecificities and have been implicated in specific HLA-disease associations, such as insulin-dependent diabetes mellitus (5–6, 19) and pemphigus vulgaris (20). Further, allelic differences of the DQB1 genes between donor and recipient in organ transplantation sometimes cause a high incidence of complications, such as rejection and organ failure (21). Therefore, a simple and accurate genotyping of the DQB region is important for routine work.

Recently, a simple and complete DQB1 genotyping by the PCR-SSO method was reported using two pairs of specific primers and 12 oligonucleotide probes (16), but it still needs separate hybridizations by each isotope-labelled probe. A non-radioactive "reverse dot blot" method has been used for SSO typing in the DQA1 gene region (22), but the amount and length of oligonucleotide probes must be adjusted and suitable conditions for hybridization determined, which is technically demanding.

In contrast, our modified PCR-RFLP method for DQB1 genotyping, depending mainly on cleavage or uncleavage of the amplified segment by informative restriction enzymes, provides a much simpler technique than the PCR-SSO method, and gives more accurate results than our previous PCR-RFLP methods (11). Two alleles of DQB1*0501 and DQB1*0503 or DQB1*0302 and DQB1*0303,

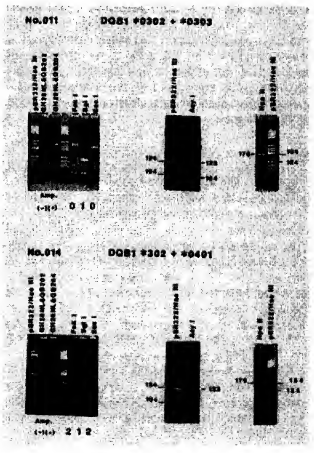

Figure 3. Modified PCR-RFLP typing for the DQB1 gene with the DQw2,3,4 alleles amplified by DQw2,3,4-specific primers (GH28NL and QB204) using DNAs of normal Japanese. Determination of their genotypes is based on the combinations given in Tables 3 and 4. All of the DQw2,3,4 alleles have a cleavage site for Sac I at the 14th nucleotide position from the 3′ terminal and so, in case of Sac I digestion, 223 bp fragment-positive alleles are indicated by 0 for simplification (see text).

which could not be defined by our previous method (11), were clearly typed even for heterozygotes by this modified PCR-RFLP method. Thus, complete DQB1 genotyping, including homozygotes and heterozygotes, can be unequivocally defined by the restriction enzymes selected here, except for discrimination between DQB1*0602 and DQB1*0603. For discrimination between these, the PCR-SSO method, as well as analysis of DR-DQ linkage disquilibrium, can be used to complement this method, if necessary (in the Japanese population, DQB1*0603 is very rare, less than 1%).

This modified PCR-RFLP method saves time in typing because the amplification conditions are the same for two pairs of specific primers and enzyme digestion is complicated within 3 h or less. The whole procedure takes 7 h after DNA has been extracted. All of the restriction enzymes used here are commercially available, and the expensive ones (e.g. SfaN I) can be replaced by other cheaper restriction enzymes described in our previous paper (11) (K. Kobayashi et al., personal communication), or omitted, depending on the typing sensitivity required.

In conclusion, this modified PCR-RFLP method for routine DQB1 typing is technically simple and rapid, eliminating the need for radioactive or non-radioactive SSO probes, and can be substituted for conventional serological and cellular typing. This modified PCR-RFLP typing, combined with several group-specific primers, has been successfully extended to HLA-DRB1 genotyping (M. Ota et al., submitted).

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